



RESEARCH ARTICLE

Spectrophotometric Calibration and Comparison of Different Semen Evaluation Methods in Nili-Ravi Buffalo Bulls

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ABSTRACT

This study was designed with the aim to find suitable spectrophotometric wavelength for accurate measurement of sperm concentration in Nili Ravi buffalo and to compare different quantitative methods (visual, hemocytometric and spectrophotometric) for the assessment of sperm concentration. A total of 78 fresh semen ejaculate were collected from breeding buffalo bulls, diluted with required concentrations and relationship between sperm number and absorbance was determined at 350, 450, 546 and 650 nm wavelengths by using spectrophotometer. The spectrometric results showed that λ_{max} was obtained at 350 nm. The derived equation between sperm concentration counted by haemocytometer and absorbance at 350 nm was $Y = 0.1135x + 0.0002$ ($R^2 = 0.9924$), at 450 nm, $Y = 0.1011x - 0.0436$ ($R^2 = 0.9756$), at 546nm, $Y = 0.0825x - 0.0101$ ($R^2 = 0.9603$) and at 650 nm, $Y = 0.0774x - 0.0235$ ($R^2 = 0.9602$). These results were later compared with relative sperm numbers at different dilutions. The results showed that 350nm wavelength appeared suitable for estimation of sperm concentration compared to others. Additionally, it was found that both photometer ($P < 0.01$) and haemocytometer ($P < 0.01$) significantly counted more sperm cells than visual assessment as used regularly on different SPUs. Moreover, haemocytometer counted significantly (834×10^6 Vs 678×10^6 , $P = 0.005$) more sperm cell than Photometer. Finally, regression analysis between haemocytometer and Photometer showed significant slope of regression and regression coefficient was 0.771 which warns that photometer must be calibrated before proper employment for routine measurement of sperm concentration.

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INTRODUCTION

Buffalo offers significant contribution in Asian livestock as more than 96% of the population is distributed across Asia including Pakistan (Afzal and Naqvi, 2004; Raziq *et al.*, 2010). In Pakistan average buffalo population has been increased from 37.7 million (2016-2017) to 38.8 million (2017-2018) with milk increase from 34,122 tons (2016-2017) to 35,136 tons (2017-2018) (Anonymous, 2017; Anonymous, 2018).

Among multiple reproduction biotechnologies, artificial insemination (AI) is the only technique that has been widely practiced in Pakistan for the improvement of

genetics and reproduction on farms (Warriach *et al.*, 2015). AI ensures efficient use of bull semen when single ejaculate is split into number of doses instead of natural mating where a bull is used on limited number of female animals (Heise *et al.*, 2011; Farooq *et al.*, 2013; Mustafa *et al.*, 2018). But number of sperm cell inseminated is an important factor for the effectiveness of artificial insemination (Fiaz *et al.*, 2010; Shoaib *et al.*, 2014). Consequently, appropriate estimation of sperm cell concentration per ejaculate is important in AI (Gaviraghi *et al.*, 2013; Yimer *et al.*, 2014).

Different procedures are used to determine the sperm cell concentration such as conventional counting, manual

or photometer counting. Practically, in many SPUs, sperm cell concentration is assessed by visual method that is usually based on color, opacity (clarity), volume and turbidity (swirling). Determination of sperm concentration for semen evaluation is an important step carried out by using haemocytometer but variations exist because of duplicate counting by same technician or different counting by different technicians. Haemocytometer is used for standardization of spectrophotometer which is usually used by many AI stations (Atiq *et al.*, 2011; Sohail *et al.*, 2013). For this reason, proper assessment of sperm concentration is important to attain the desired calibration of the instrument, because it would ensure the number of sperm cells per insemination dose (Eljarah *et al.*, 2013).

Spectrophotometer is routinely optimized and standardized with haemocytometer and a standard curve is developed by spectrophotometric absorbance of sperm cells that gives correct results in short time. Development and use of standard curve have been reported in different species of cattle (Prathalingam *et al.*, 2006) fishes (Ereene *et al.*, 2010; Zhang *et al.*, 2014) and in mussel (*Mytilusedulis* L) (Miguel *et al.*, 2008). In domestic animals, Photometer with specific wavelength has been used for the estimation of sperm concentration in various cattle species. Atiq *et al.* (2011) used photometer with 546 nm wavelength for sperm concentration estimation in cattle. Prathalingam *et al.* (2006) and Andrabi *et al.* (2002) also used photometer at 685 for cattle and 546nm respectively for crossbreed (Friesian and Sahiwal) bulls. However, no study is present in buffaloes for sperm estimation.

Thus, this study was designed with aims (1) To find suitable spectrophotometric wavelength for accurate measurement of sperm concentration in Nili Ravi buffalo and (2) Comparison of different quantitative methods (visual or conventional, haemocytometer and photometric) for the assessment of sperm concentration.

MATERIALS AND METHODS

Sample collection: A total of 78 semen ejaculates were collected randomly from buffalo bulls that were housed under uniform feeding conditions and management at Al-Haiwan Sires, Sahiwal. Each bull was sexually stimulated and pre-warmed (42°C) artificial vagina was used to collect ejaculate from each animal and later shifted immediately to semen evaluation room. Each semen sample was observed for its color, volume, consistency, concentration and sperm motility.

Spectrophotometric wavelength determination for sperm concentration assessment in Nili Ravi buffalo: For each sample type different dilutions (semen: saline water) 1:250, 1:200, 1:150, 1:100, 1:50 and blank were prepared. Spectrophotometer was switched auto zero by using normal saline water as reference. For five dilutions of each sample, absorbance spectrum was obtained from 350 to 850 nm wavelength with interval of 1nm by using UV / Visible double beam spectrophotometer (Bio-Med, 1602, Canada). Readings from the spectrophotometer were used to detect the maximum absorbance wavelength (Knox *et al.*, 2003).

Standard curve establishment between sperm concentration and absorbance: Absorbance was measured for each dilution at different wavelength and sperm cell count of original sample was found. A linear relationship was established for each sample and then equation between sperm concentration and absorbance was constructed through pooling the statistics from all samples and sample collection procedure (Ereene *et al.*, 2010).

Semen assessment by quantitative methods

Conventional evaluation: In field, usually visual counting is used as conventional evaluation based on color, opacity (clarity), volume and turbidity or swirling (Knox *et al.*, 2003). The visual counting includes mass motility guided by estimation the wave pattern of sperm cell (4×) as well as sperm motility (10×) under compound microscope as presented in Table 1.

Table 1: Different dilutions for the preparation of semen doses in field conditions

Sr. No.	Volume	Mass Motility	Dilution (ml)
1	1ml	++++	20
2	1ml	+++ (+)	17
3	1ml	+++	10
4	1ml	++ (+)	07
5	1ml	++	05
6	1ml	+ (+)	03
7	1ml	+	01

[++++ = Four plus sample, +++ (+) = Three and half plus sample, ++ = Three plus sample, ++ (+) = Two and half plus sample, + = Two plus sample, + (+) = one and half plus sample, + = One plus sample].

Haemocytometer evaluation: A 20µL semen sample was mixed in 2 ml of formal saline to fix the sperm cells. Coverslip was placed at the central area of haemocytometer. A 10µL sample was taken and micropipette was placed close to edge of cover slip and sample was uniformly distributed in chamber by capillary action. Sperm cell counting was performed at 40× with compound microscope after the sperm cells had completely settled within the counting chamber (about 2-10 min after loading the sample). Spermatozoa head in five squares of each hemocytometer chamber were counted. Sperm cell numbers per ml was calculated by following formula;

Cell concentration (cells/ml) = Number of cells count in 5 chambers × 5 × 10,000 × 100

Photometric evaluation: By using photometer, sperm cell concentration was determined at 535 nm wave length by using pre-warmed and calibrated photometer. Photometer was set at zero by using normal saline water and then 20 µL semen samples was mixed in 2 ml of normal saline water (Atiq *et al.*, 2011).

Statistical analysis: Data analysis was performed by using SYSTAT 12 (Systat) and Microsoft Excel 2007 version. All the datafiles were accessed using Microsoft Excel. Correlation analysis was performed between absorbance and sperm concentration, and a linear relationship of sperm concentration determined by

hemocytometer with the absorbance of spectrophotometer was represented by the coefficient of determination (R^2 -value) given by software after the standard curve was developed (Fry, 1993).

RESULTS

Suitable wavelength determination in Nili Ravi buffalo: Initially, spermatozoa spectrum (200-850 nm) obtained from all buffalo semen samples exhibited similar pattern for all semen dilutions when absorbance values were under 1.5 as shown in Fig. 1 (A). Generally, progressive linear reduction in absorbance with the increase in wavelength was observed. At low wavelength, linear relationship of absorbance for high sperm concentration was disrupted by a peak. But wide variations in absorbance values were also observed at low wavelength when spectrum was obtained at range between 200-850 nm wavelengths. So, in further experiment wavelength range was set between 350-850 nm as shown in Fig. 1b and four wavelengths 350, 450, 546 and 650 nm

were selected for the construction of equation for sperm concentration. Absorbance values above 1.0 and below 0.1 were eliminated from consideration for regression analysis.

Regression analysis also represented a decrease in regression coefficient (slope) with an increase in wavelength as shown in Fig. 1. As, spermatozoa varied at higher wavelength that's why inverse of regression coefficient ($1/b$) was measured (Table 2). The regression coefficient was determined as change in each unit of optical density (O.D) or absorbance leading to great variations in sperm cell concentration assessment at higher wavelength.

Results indicated that not a single maximum absorbance peak was observed in visible range that's why any of four wavelengths with high absorbance values were appropriate for the generation of standard curve. At shorter wavelength higher absorbance values for buffalo semen samples were observed than longer wavelength. Therefore, wave length of 350 nm was selected for calculations and the generation of standard curve.

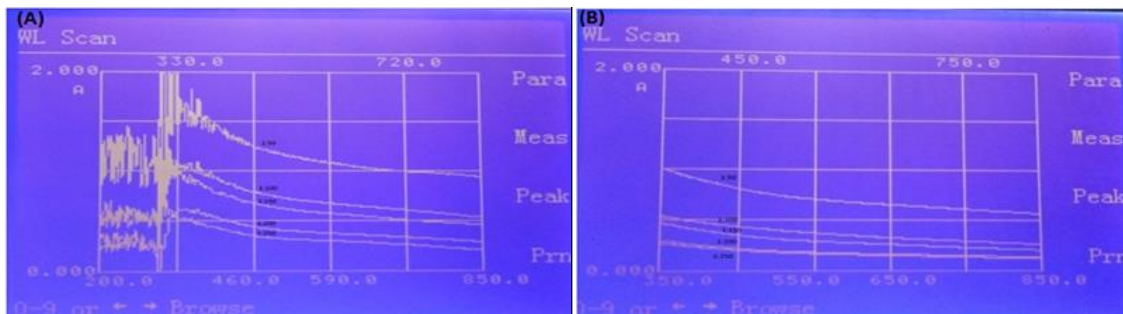


Fig. 1: Serial dilution spectrum of Nili-Ravi buffalo semen samples (A) 200-850 nm Wavelength (B) 350-850 nm Wavelength

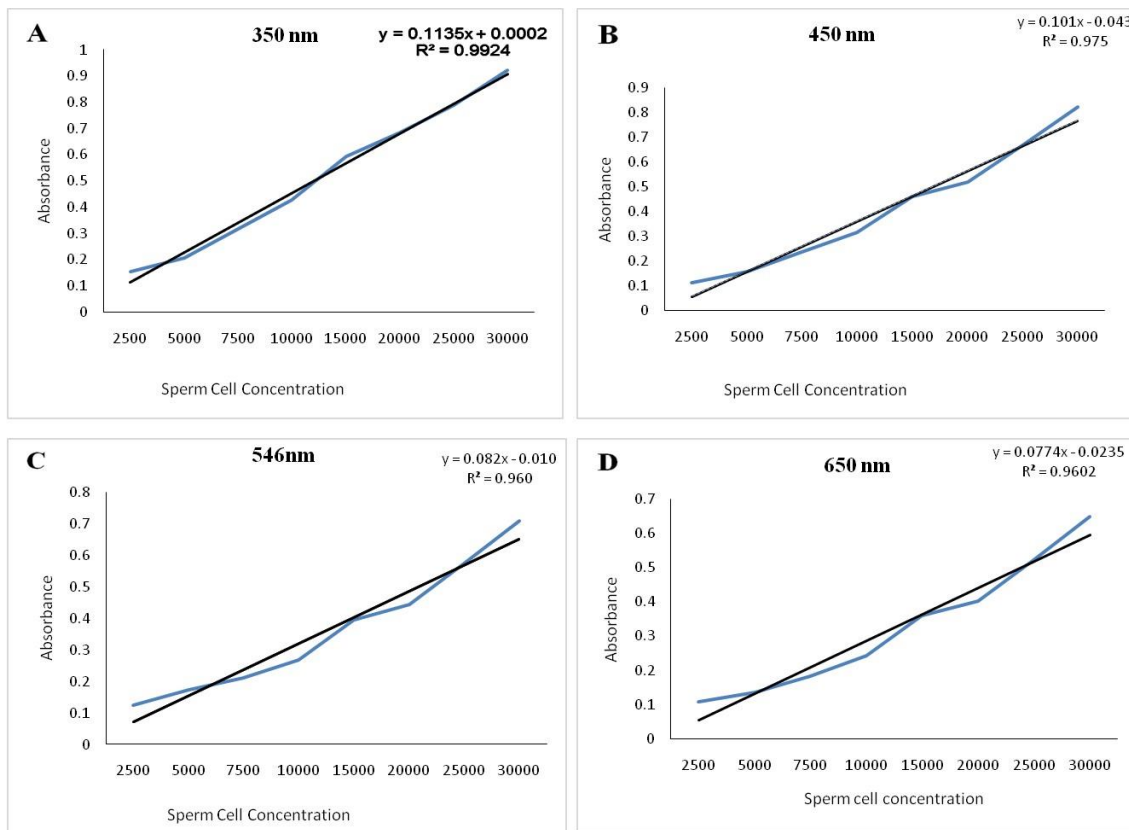


Fig. 2: Standard calibration curves for Nili-Ravi buffalo semen samples at (A) 350nm (B) 450nm (C) 546nm (D) 650nm wavelength.

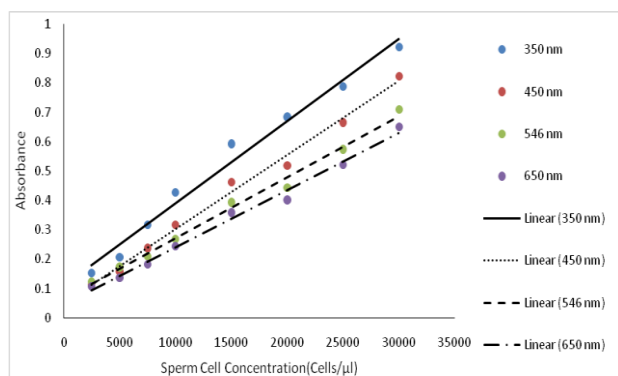


Fig. 3: Regression lines between sperm concentration and absorbance (values between 0.1-1) at different wavelength 350, 450, 546 and 650 nm.

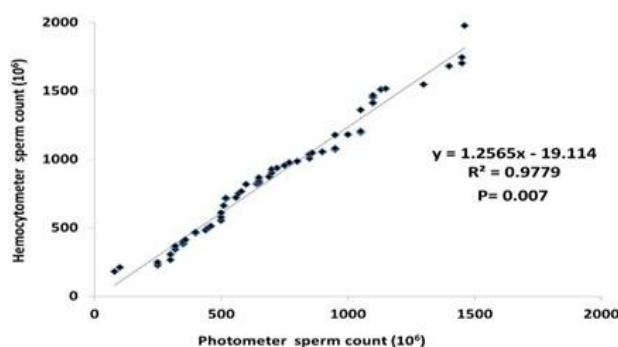


Fig. 4: Linear least square coefficient analysis of sperm concentration measured by Haemocytometer and Photometer.

Table 2: Regression analysis parameters: Coefficient of determination (R^2), intercept (a) and regression coefficient (b) at Different Wavelength in Nili-Ravi Buffalo

Wavelength (nm)	Intercept (a)	Regression coefficient (b)	1/b	Coefficient of determination (R^2)
350	0.0002	0.1135	8.810	0.9924
450	0.0436	0.1011	9.891	0.9756
546	0.0101	0.0825	12.121	0.9603
650	0.0235	0.0774	12.92	0.9602

Table 3: Average sperm concentration (10^6) in Nili-Ravi Buffalo bulls measured by Visual, Hemocytometer and Photometer

Sperm Counting Methods	Mean \pm SD (10^6)
Mass motility grading	402.1 \pm 112.8 ^c
Hemocytometer	834.6 \pm 341.7 ^a
Photometer	678.6 \pm 297.7 ^b

Standard curve for correspondence between sperm concentration and optical density: A significant linear correspondence was observed between sperm cell concentration and optical density at different wave length i.e 350, 450, 546 and 650 nm (Fig. 2) for all serial dilution of buffalo bull semen samples (n=78). For standard curve, effective optical density values range between 0.100-1.00 and maximum absorbance was observed at 350nm as shown in Fig. 2(A). The derived equation between spermatozoa concentration and absorbance at 350 nm was $Y = 0.1135x + 0.0002$, at 450 nm $Y = 0.1011x - 0.0436$, at 546 nm $Y = 0.0825x - 0.0101$ and at 650 nm $Y = 0.0774x - 0.0235$ where X is the optical density or absorbance values while Y is the sperm cells concentration (cells/ μ L) as shown in Fig.2 (A, B, C, D). The coefficient of determination at different wave length was $R^2 = 0.9924$ at 350 nm, $R^2 = 0.9756$ at 450 nm, $R^2 = 0.9603$ at 546 nm and $R^2 = 0.9602$ at 650 nm wavelength (Table 2).

Comparison of various quantitative methods for sperm cell determination: The results showed that both photometer and haemocytometer significantly counted more sperm cells than visual assessment as presented in Table 3 and 4. Moreover, haemocytometer counted significantly higher sperm concentration than that of Photometer. Ultimately, regression analysis between Photometer and haemocytometer demonstrated significant slope of regression and regression coefficient was 0.771 (Fig. 4). As represented in Table 4 semen production units can improve ~30% of its production by the use of carefully calibrated photometer in Nili-Ravi buffalo bulls.

DISCUSSION

In artificial insemination program, spermatozoa quantification is the influencing factor to optimize fertility. AI program require adequate determination of sperm concentration because fairly low number of sperm cells per insemination unit are used without depression in fertility. Due to recent trends towards heightened awareness of all aspects of reproduction, many commercial farmers offer special emphasis on the quality parameters of semen doses in the sire selection process. For this, the animal breeding stations use multiple methods of determining sperm concentration for uniform and quality preparation of insemination doses for their customers (Anzar *et al.*, 2009). Spectrophotometric evaluation of sperm concentration is commonly used practice that directly influences the productivity of AI breeding centers as reported by Atiq *et al.* (2011) and Knox *et al.* (2003). Spectrophotometric instruments are routinely re-calibrated and optimized with highly sensitive methods like hemocytometer that utilized to construct standard curve between sperm concentration and absorbance (Anzar *et al.*, 2009). The study is perhaps the first study in buffalo, in which spectrophotometric investigation was used to determine sperm concentration in fresh semen sample. The study investigated spectrophotometric investigation to acquire spectrum absorbance for buffalo bull semen samples and found that specific range of wavelength with higher absorbance peaks would be better choice. This comes to an agreement with Ereene *et al.* (2010) as no maximum peak was found, so single continuous scan of visible absorbance spectrum was obtained with the interval of 1nm, Simultaneous decrease in absorbance values with increased wavelength was also noticed in present study that's why separate testing for suitable wavelength was not needed as reported in previous studies (Miguel *et al.*, 2008; Ereene *et al.*, 2010). Previous studies used wavelength range of 500-600 (Foote, 1978) and 546nm (Atiq *et al.*, 2011) for cattle, therefore sperm concentration was determined with absorbance values in these range values. The results however, determined that 350nm wavelength in which λ_{max} was obtained with higher R^2 value (0.9924) appeared better than other wavelengths. We expect that the difference in our study with that of other studies in cattle is obviously due to species difference, however it motivates that photometer with an alternative wavelength must be examined for optimization of buffalo bull sperm counting.

Table 4: Comparison of different quantitative methods (Visual, Hemocytometer and Photometer)

Parameters	Visual counting	Hemocy-tometer	Photo-meter
Number of ejaculates	78	78	78
Average sperm concentration / mL	402.1	834.6	678.6
Number of doses / ml @ 50 × 10 ⁹ /ml	8	16	14
Production level of a SPU: 20 bulls, 4 mL each	640	1280	1120

In the second part, different methods for sperm counting were compared calculating fresh semen concentration in Nili-Ravi buffalo bulls at different SPUs. Interestingly, we found significant difference in sperm counting among all counting methods. It is understandable that dilution of fresh semen by using mass motility grading is a raw method that needs to be discontinued among all SPUs. On the other hand, haemocytometer cannot be used in routine for semen evaluation in AI laboratory as it is time consuming method and is unable to efficiently evaluate large number of semen samples (Cadena-Herrera *et al.*, 2015). Due to the difference between counting by haemocytometer and photometer in this study, we draw a correlation and found that commercial photometers must be routinely calibrated with that of haemocytometer because significant variations existed when sperm numbers of a sample was more than 1.5 billion/ml and that is the reason that significance was found between these two methods. Another reason of difference is that commercial photometers as in current study (535 nm) are mostly manufactured for that of cattle semen assessment and therefore, this warns that species difference must take into account before using photometer.

Conclusion: The present study found that for fresh buffalo semen samples, 350 nm wavelength appears better for sperm counting which will facilitate the optimization and standardization of the spectrophotometric methodology in buffalo bull furthermore, it warned that sperm counting with that of mass motility grading should be avoided and careful calibration of spectrophotometer with haemocytometer is helpful for measuring sperm concentration.

Authors contribution: HR conceived the idea, SR executed the research plan, SR and HR wrote the paper, LS, QL, JLZ, MRY revised and finally approved the manuscript.

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